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Histone Acetylation in Keratinocytes Enables Control of the Expression of Cathelicidin and CD14 by 1,25-Dihydroxyvitamin D_3

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Hormonally active vitamin D_3—1,25-dihydroxyvitamin D_3 (1,25D3)—acts as a signaling molecule in cutaneous immunity by increasing pattern recognition through Toll-like receptor-2 (TLR2), and increasing the expression and function of antimicrobial peptides. Here we show that the actions of 1,25D3 on keratinocyte innate immune responses are influenced by histone acetylation and require the steroid receptor coactivator 3 (SRC3), which mediates inherent histone acetyltransferase (HAT) activity. SRC3 was detected in the suprabasal and granular layer of the skin, similar to cathelicidin expression. HAT activity was important to keratinocyte cathelicidin expression as the combination of histone deacetylase inhibitors (HDACi) (butyrate or trichostatin A) and 1,25D3 increased cathelicidin and CD14 expression and enhanced the antimicrobial function of keratinocytes against *Staphylococcus aureus*. This treatment, or activation of TLR2, also directly increased acetylation of histone 4. Small interfering RNA silencing of the vitamin D receptor or SRC3 blocked the induction of cathelicidin and CD14 by 1,25D3. HDACi could not reverse this effect or influence cathelicidin in the absence of 1,25D3, suggesting that both are necessary for function. These studies demonstrate that the epigenetic control of gene transcription by histone acetylation is important for 1,25D3-regulated antimicrobial and TLR function of keratinocytes, essential elements of the innate immune response of the skin.

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INTRODUCTION

The innate immune system comprises a multiplicity of evolutionary ancient host mechanisms that are activated to enable defence against a broad spectrum of microbial threats. While current therapy of infectious skin disease generally targets the pathogen, new treatment options are being considered that could treat or prevent infection by influencing innate immune function. Since traditional treatment with antibiotics fails in an increasing number of patients due to development of bacterial resistance, this approach might become highly attractive in infectious and inflammatory skin disease. Understanding the regulatory pathways and signaling molecules involved in the control of the cutaneous innate immune system is critical for this process.

The active metabolite of vitamin D_3—1,25-dihydroxyvitamin D_3 (1,25D3)—has recently been identified as an important signaling molecule controlling innate immune responses in keratinocytes. In skin injury, activated vitamin D_3 metabolism leads to rapid induction of genes important for microbial recognition and antimicrobial defense (Schauber *et al.*, 2007); in particular, 1,25D3 induces CD14 and Toll-like receptor-2 (TLR2) in keratinocytes enabling increased response to TLR activation (Schauber *et al.*, 2007). Simultaneously, vitamin D_3 induces antimicrobial cathelicidin peptide expression strengthening the chemical antimicrobial shield produced by keratinocytes (Wang *et al.*, 2004; Weber *et al.*, 2005; Schauber *et al.*, 2006). In clinical studies, topical treatment with 1,25D3 increases cathelicidin (Weber *et al.*, 2005) and TLR2 expression in healthy skin suggesting a role for vitamin D_3 or its analogs as topical immune modulators (Schauber *et al.*, 2007).

The genomic effects of 1,25D3 are mediated by its nuclear hormone receptor, the vitamin D receptor (VDR). After binding of 1,25D3, the VDR binds to consensus sequences called vitamin D-responsive elements in the promoter of target genes such as cathelicidin (Wang *et al.*, 2004). The VDR subsequently recruits different coactivators to initiate transcriptional activity. It was recently demonstrated that

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Abbreviations: 1,25D3, 1,25-dihydroxyvitamin D_3; HAT, histone acetyltransferase; HDACi, histone deacetylase inhibitors; NHEK, normal human epidermal keratinocyte; SRC, steroid receptor coactivator; TLR Toll-like receptor; TSA, trichostatin A; VDR, vitamin D receptor

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the VDR binds to coactivator complexes such as DRIPs (VDR-interacting proteins also called mediator) or SRCs (steroid receptor coactivators) during keratinocyte differentiation (Oda *et al.*, 2003, 2007; Hawker *et al.*, 2007). During this process, the coactivators have different functions. In proliferating keratinocytes, DRIP binding to the VDR dominates, whereas in differentiated keratinocytes SRC binding is preferred (Oda *et al.*, 2003). Furthermore, different genes prefer one type of complex compared to the others. For example, deletion of DRIP205 results in marked inhibition of keratin 1 expression, whereas deletion of SRC2 has little effect. In contrast, loricrin expression is highly dependent on DRIP205 as well as SRC2 and -3 (Hawker *et al.*, 2007). The coactivator complexes have been shown to cycle on and off nuclear hormone response elements such as vitamin D-responsive elements in alternating patterns, suggesting that both types of complexes are required for optimal gene regulation. DRIP205 binds directly to VDR, and the DRIP complex contains components of the transcription initiation complex linking it directly to the RNA polymerase machinery (Rachez *et al.*, 2000). SRC family members such as SRC3 form very different complexes with VDR. In particular, they recruit a number of histone acetyltransferases (HATs) such as CBP/p300, which by increasing histone acetylation open up the chromatin, thus facilitating access of transcription factors to the transcription start site (Leo and Chen, 2000). Reversible acetylation of histone proteins is generally correlated with active transcription, whereas deacetylation is linked to transcriptional repression.

Histones H2A, H2B, H3, and H4 are the core subunits that comprise the nucleosome, the smallest unit of eukaryotic chromatin. The N termini of these four histones are accessible to post-translational modifications directly influencing gene transcription. Both histone tails and globular domains are subject to post-translational modifications, and these modifications have different functions (reviewed in Li *et al.*, 2007). The modifications include methylation of arginine residues; methylation, acetylation, ubiquitination, ADP ribosylation, and sumoylation of lysines; and phosphorylation of serines and threonines (Li *et al.*, 2007). With the exception of methylation, histone modifications result in a change in the net charge of nucleosomes, which could loosen inter- or intranucleosomal DNA–histone interactions. Increased histone acetylation at the promoter region has been linked to active transcription (Workman and Kingston, 1998). Recently, Pokholok *et al.* (2005) demonstrated that increased acetylation of H3 and H4 correlates with increased transcription rate. The locations of these chromosomal modifications are tightly regulated by HATs, such as the SRC3/p300 complex and histone deacetylases (HDACs), and are crucial for their effect on transcription.

The short-chain fatty acid butyrate and trichostatin A (TSA) increase acetylation of histone H4 by inhibition of HDAC activity. This in turn results in induction or repression of genes in various cells including keratinocytes (Saunders *et al.*, 1999). Changes in histone acetylation have been considered to ''shape'' the response to additional stimuli such as growth factors (Wang *et al.*, 1992). These data on epigenetic control of gene transcription by histone modifications led to our hypothesis that changes in histone acetylation could influence the expression and function of innate defense genes in keratinocytes, modifying their regulation by 1,25D3. In this study, we demonstrate that increased histone acetylation amplifies the effect of 1,25D3 on innate immune function of human keratinocytes. These observations offer direct evidence of epigenetic control of keratinocyte immune defense function.

RESULTS

The effects of vitamin D_3 on keratinocyte innate immune function suggested that VDR coactivators such as SRC3 and DRIP205, which participate in distinct ways in mediating vitamin D-dependent keratinocyte differentiation, may also be involved in regulation of cutaneous innate immunity. We first evaluated the localization of DRIP205 and SRC3 in human skin using immunohistochemistry. DRIP205 was present throughout the epidermis (Figure 1a). The expression of SRC3 was distinct from DRIP205. SRC3 was strongly expressed in the differentiated keratinocytes in the superficial epidermis with expression increasing gradually from spinous to granular layer (Figure 1b). Also, while DRIP205 staining was mainly nuclear, SRC3 was detected in both cytoplasm and the nucleus in keratinocytes. This expression of the VDR coactivator SRC3 corresponded closely with the previously reported expression of cathelicidin in human epidermis after topical treatment with 1,25D3 (Schauber et al., 2007) and is consistent with observations showing that cathelicidin antimicrobial peptide (hCAP18/LL-37) is induced by the activity of 1,25D3 (Wang *et al.*, 2004). Thus, SRC3 and cathelicidin show a similar expression pattern that is distinct from another VDR coactivator, DRIP205.

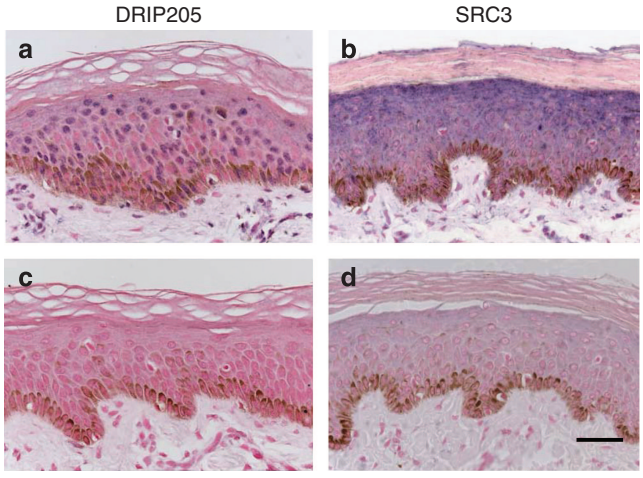

Figure 1. SRC3 expression is localized to skin layers that express 1,25D3-dependent innate defense genes. Differential localization of DRIP205 (**a**) and SRC3 (**b**) in the epidermis. Human adult skin sections were incubated with antibodies against DRIP205 and SRC3, and the signals were visualized with biotinylated secondary antibody and NBT/BCIP staining (purple/blue color). The nuclei were counterstained with nuclear fast red (pink color; DRIP205 and SRC3). DRIP localizes to nuclei in the basal and suprabasal layer, whereas SRC3 is expressed in differentiated keratinocytes in the outmost epidermis. (**c** and **d**) Corresponding sections stained with the respective preimmune control antibodies. Bar = 35 μM.

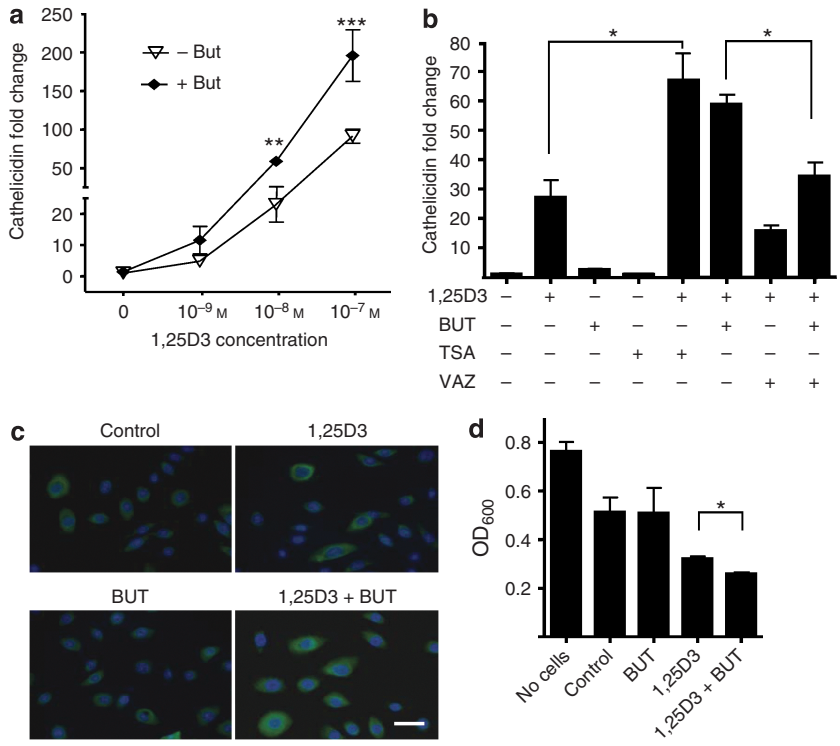

Figure 2. HDAC inhibition increases 1,25D3-mediated induction of cathelicidin antimicrobial peptide in human keratinocytes. (**a**) NHEKs were stimulated with 1,25D3 (10^{-9}–10^{-7} M) in the presence of the HDAC inhibitor butyrate (BUT; 2 mM) for 24 hours. mRNA abundance was determined by real-time qPCR for cathelicidin and glyceraldehyde-3-phosphate dehydrogenase and normalized to vehicle-treated controls. BUT alone had no effect, whereas HDAC inhibition strongly increased 1,25D3-induced cathelicidin. Data shown are means (±SD) of the results from a single stimulation experiment performed in triplicates and are representative of at least three independent experiments (**$P<0.01$; ***$P<0.001$; two-way analysis of variance). (**b**) The HDAC inhibitor TSA (200 ng ml^{-1}) induces cathelicidin in the presence of 1,25D3 (10^{-8} M). Expression of cathelicidin is dependent on a functional VDR, as pretreatment with the VDR antagonist ZK159222 (10^{-7} M) inhibits induction of cathelicidin with BUT (2 mM) in the presence of 1,25D3 (10^{-8} M) after 24 hours. Expression of cathelicidin was determined by qPCR as described in **a**. (*$P<0.05$; Student's *t*-test). (**c**) Increased transcript correlates with enhanced cathelicidin protein expression after HDAC inhibitor and 1,25D3 stimulation. NHEKs grown on chamber slides were stimulated with the vehicle, 1,25D3 (10^{-8} M), BUT (2 mM), or the combination for 24 hours. Cells were stained with a polyclonal anti-LL-37 antibody (followed by an FITC-coupled secondary antibody) and nuclei detected with 4,6-diamidino-2-phenylindole (Bar = 30 μM). (**d**) Enhanced antimicrobial activity of keratinocytes after BUT and 1,25D3 stimulation. NHEKs were stimulated with 1,25D3 (10^{-8} M) in combination with the HDAC inhibitor BUT (2 mM) for 24 hours, cells harvested and cell lysates coincubated with *S. aureus* ΔmprF, and bacterial growth monitored over time to determine antimicrobial activity. Media not containing cell lysates or containing cell lysates from unstimulated cells were used as controls. OD_{600} readings after 6 hours incubation are displayed (*$P<0.05$; Student's *t*-test).

SRC3 complexes possess HAT activity unlike DRIP complexes. To investigate if histone acetylation influences antimicrobial peptide expression in skin, normal keratinocytes were treated with butyrate to increase histone acetylation by inhibition of HDACs. Cells were exposed to increasing 1,25D3 levels at a constant butyrate concentration (2 mM) and the expression of cathelicidin (hCAP18/LL-37) mRNA measured by quantitative real-time PCR (Figure 2a). Unlike the previously reported response of colonic epithelial cells to butyrate (Schauber *et al.*, 2003, 2004), the addition of butyrate alone did not change cathelicidin transcript abundance in keratinocytes. However, HDAC inhibition did significantly amplify cathelicidin expression in keratinocytes in the presence of 1,25D3. In the presence of 10^{-7} M 1,25D3, cathelicidin increased over 200-fold in the presence of butyrate but only ca. 75-fold in its absence. Twenty-four hours after treatment with butyrate alone or in combination with 1,25D3, no change was seen in cell morphology, cell death, or apoptosis as measured by microscopic inspection, LDH release, and annexin V/propidium iodide staining, respectively (data not shown). TSA—another HDAC inhibitor structurally unrelated to butyrate—showed similar effects and also significantly increased 1,25D3-induced cathelicidin (Figure 2b). Treatment with the VDR antagonist ZK159222 decreased the capacity of the HDAC inhibitor to increase cathelicidin (Figure 2b), thus suggesting that increasing histone acetylation will aid VDR transcriptional activity but that cathelicidin expression was still dependent on the action of the VDR. In addition to the increase in mRNA, an increase in cathelicidin peptide expression was seen by immunostaining of keratinocytes treated with butyrate and 1,25D3 (Figure 2c). Furthermore, HDAC inhibition increased the inherent antimicrobial activity of 1,25D3-stimulated keratinocytes. Protein extracts from cells treated with 1,25D3 and butyrate displayed maximal ability to suppress the growth of *Staphylococcus aureus* ΔmprF (Figure 2d). Histone deacetylase inhibitors (HDACi) and 1,25D3 did not show antimicrobial activity themselves (data not shown). These findings are

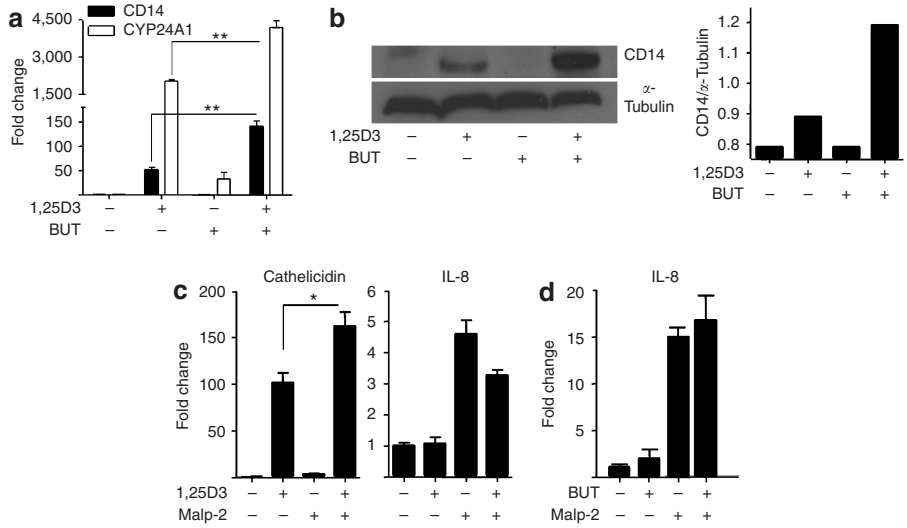

Figure 3. HDAC inhibition in keratinocytes increases CD14, CYP24A1, and cathelicidin, but not IL-8. (**a**) NHEKs were stimulated with 1,25D3 (10^{-8} M), in the presence of butyrate (BUT; 2 mM) for 24 hours. Transcript abundance of 1,25D3-regulated CD14 and CYP24A1 was determined by qPCR (**$P<0.01$; Student's *t*-test). (**b**) (Upper panel) Western blot of CD14 protein expression in NHEK stimulated with control (lane 1), 1,25D3 (10^{-8}M; lane 2), BUT (2 mM; lane 3), or the combination of 1,25D3 and BUT (lane 4) for 24 hours. A band at approximately 50 kDa was detected corresponding to CD14 protein. Membranes were reprobed with an anti-α tubulin antibody and protein abundance quantified by densitometry (right panel). (**c**) NHEKs stimulated with 1,25D3 (10^{-8} M), the TLR2/6 ligand Malp-2 (0.1 μg ml^{-1}), or the combination, and cathelicidin or IL-8 transcript abundance determined by qPCR (*$P<0.05$). (**d**) HDAC inhibition by BUT did not enhance IL-8 transcript induced by Malp-2.

consistent with an increase in proteolytic activation of cathelicidin in addition to the observed increase in mRNA and protein expression. However, we cannot exclude that other factors involved in 1,25D3-enhanced innate defense of keratinocytes were also increased.

To investigate if HDAC inhibition specifically increased cathelicidin, other genes in keratinocytes that are regulated by vitamin D_3 were investigated. Similar to cathelicidin, CD14 and CYP24A1 transcript abundance was strongly upregulated by 1,25D3 stimulation and further enhanced by inhibition of HDAC with butyrate (Figure 3a). An increase in CD14 protein expression was also seen in similarly treated keratinocytes (Figure 3b).

To determine if an increase in histone acetylation would also induce other immune response genes in keratinocytes, the expression of the CXCL chemokine IL-8 was measured. Previously, it was shown that activation of TLR2/6 by Malp-2 in the presence of 1,25D3 increased cathelicidin, but Malp-2 alone had no effect (Schauber *et al.*, 2007; Figure 3c). In contrast, keratinocyte IL-8 expression increased when stimulated with only Malp-2 and was not enhanced by 1,25D3 or butyrate (Figure 3c and d). Thus, increased histone acetylation selectively enhanced expression of the 1,25D3-dependent genes, cathelicidin and CD14, but did not increase IL-8 in keratinocytes.

The abundance of acetylated histone was directly measured to confirm the inhibitory effects of butyrate and TSA on HDACs. Immunofluorescence staining demonstrated that normal keratinocytes stimulated with HDACi increased histone H4 acetylation localized to the nuclei (Figure 4a). Histone H4 acetylation was further increased when keratinocytes were stimulated with HDACi in the presence of 1,25D3, which was consistent with the observed increase in transcriptional activity. To determine if changes in histone acetylation occur upon stimulation of keratinocytes with TLR ligands, normal human epidermal keratinocytes (NHEKs) were stimulated with different TLR ligands and histone acetylation was assessed. As expected, butyrate treatment increased acetylation of histone H4 (Figure 4b, lane 2). Exposure to Malp-2 or PAM3CSK to activate TLR2/6 or TLR2/1, respectively, also increased histone acetylation (Figure 4b, lanes 3 and 4). Ligands of other TLRs including poly(I:C)-TLR3, LPS-TLR4, Flagellin-TLR5, Imiquimod-TLR7, and CLO75-TLR7/8 did not change histone acetylation in primary keratinocytes.

To examine further how cathelicidin expression is influenced by histone acetylation, the role of the VDR and its two distinct coactivators, DRIP205 and SRC3, cells were examined using siRNA silencing technology. Among multiple DRIP subunits, DRIP205 was targeted because it directly binds to VDR through its NR boxes (Lempiainen *et al.*, 2005). From the two SRC family members, SRC3 was selected because it had previously been shown to increase VDR transactivation (Hawker *et al.*, 2007). As predicted by earlier experiments with the chemical VDR inhibitor shown in Figure 2b, silencing of the VDR by small interfering RNA (siRNA) blocked induction of cathelicidin by 1,25D3 (Figure 5a) and CD14 (not shown). This effect corresponded with a ca. 70% decrease in VDR protein expression as displayed in Figure 5b. Blocking of VDR coactivator DRIP205 had no effect on 1,25D3-induced cathelicidin (Figure 5c) despite inhibition of DRIP205 expression confirmed by immunoblotting (Figure 5b). In contrast, inhibition of SRC3 expression blocked vitamin D_3-induced cathelicidin expression (Figure 5c)

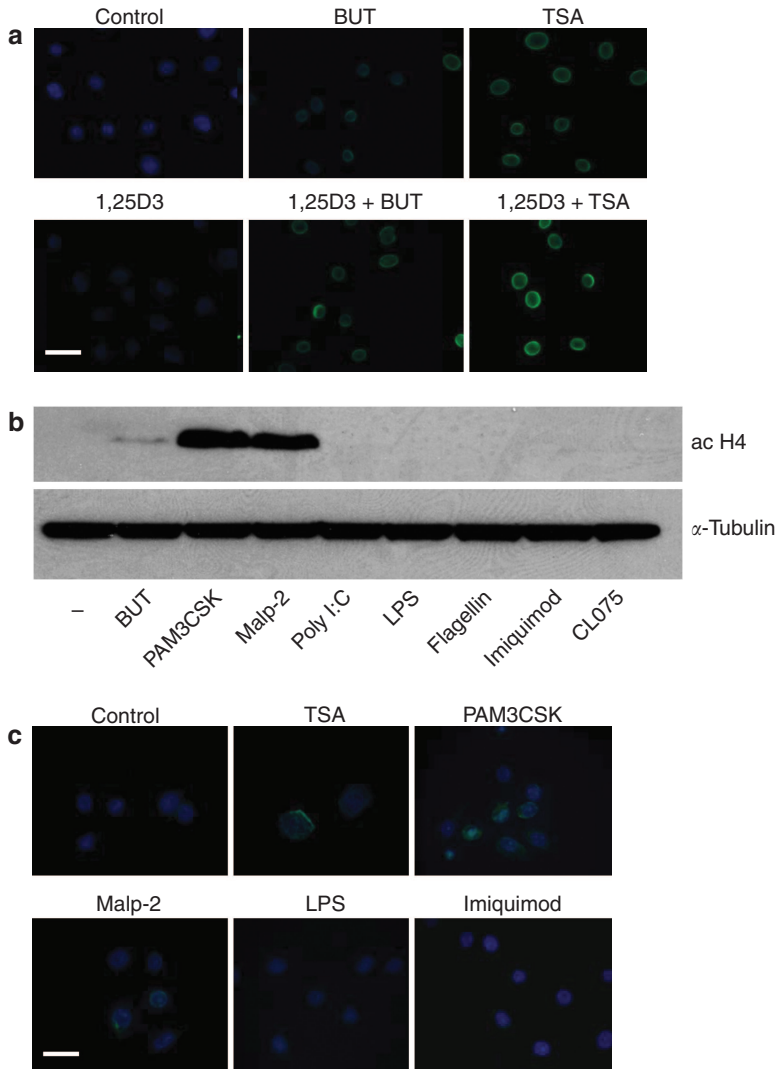

Figure 4. HDACi and TLR activation induce histone acetylation. (**a**) Immunofluorescence staining of acetylated histone H4 protein in NHEK treated with HDACi in the presence or absence of 1,25D3. Cells treated with the combination of 1,25D3 and HDACi show increased histone acetylation. (**b**) Western blot of acetylated histone 4 from keratinocytes treated with the HDACi butyrate or TLR agonists. Butyrate or stimulation with TLR2 ligands (Malp-2 or PAM3CSK) increased histone H4 protein acetylation which was confirmed by (**c**) immunofluorescence staining. Bar = 30 μM.

and CD14 (Figure 5d). Increasing histone acetylation by butyrate did not restore cathelicidin and CD14 induction by 1,25D3 when SRC3 was silenced (Figure 5d). Again, blocking DRIP205 had no effect on cathelicidin and CD14 induction by 1,25D3 in the presence or absence of HDACi (Figure 5d). These results are consistent with the HAT activity mediated by the SRC3 complex, as the inherent HAT activity of this complex would be necessary before the effects of the deacetylase inhibitor could be detected.

DISCUSSION

Vitamin D_3 has long been appreciated as a crucial factor in keratinocyte proliferation and differentiation, leading to the formation of the physical cutaneous barrier (Bikle, 2004). In addition, several studies recently uncovered an important role for vitamin D_3 in cutaneous antimicrobial defense: in cultured keratinocytes, 1,25D3 is a direct and specific inducer of cathelicidin antimicrobial peptide expression (Wang *et al.*, 2004), and induction correlates with increased antimicrobial activity against *S. aureus* (Schauber *et al.*, 2006). *In vivo,* topically applied 1,25D3 induces cathelicidin in intact human skin (Weber *et al.*, 2005; Schauber *et al.*, 2007). Furthermore, in response to TLR activation or injury, cutaneous vitamin D_3 metabolism is altered and hormonally active 1,25D3 generated to serve as a signaling molecule in a sequence of rapid defense responses in keratinocytes and monocytes (Liu *et al.*, 2006; Schauber *et al.*, 2007). The presence of 1,25D3 increases CD14 and TLR2 expression *in vitro* and *in vivo* and enables pattern recognition leading to amplification of the antimicrobial response (Schauber *et al.*, 2007). Thus, activated 1,25D3 generated in the skin or applied externally initiates an antimicrobial response and increases sensitivity to microbial challenge in skin. How this system functions in cutaneous disease states, and potential

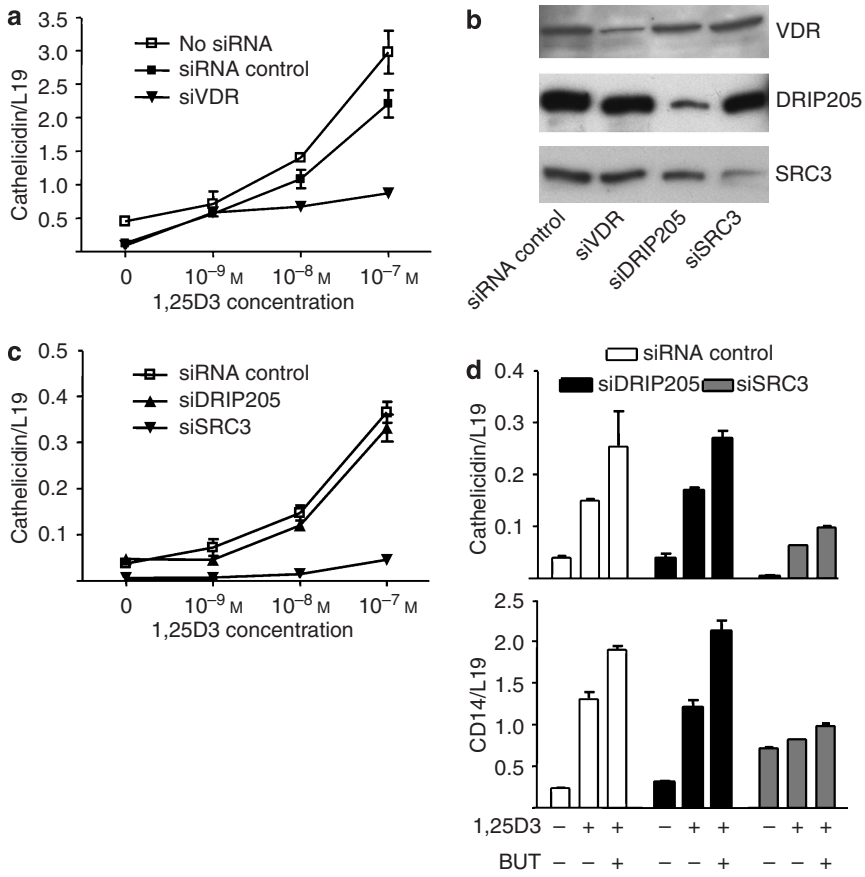

Figure 5. Increased cathelicidin by 1,25D3 is dependent on the VDR and VDR coactivator SRC3 in keratinocytes. (**a**) Silencing of the VDR blocks cathelicidin induction in keratinocytes. NHEKs were transfected with siRNA oligonucleotides for VDR, DRIP205, SRC3, or control siRNA and stimulated with increasing concentrations of 1,25D3. Expression was normalized to the housekeeping gene L19, which is unaffected by siRNA transfection. (**b**) Efficiency of siRNA silencing was evaluated by western analysis. (**c**) siRNA suppression of the VDR coactivator SRC3, but not DRIP205, blocked induction of cathelicidin by 1,25D3. (**d**) NHEK transfected with siRNA oligonucleotides for SRC3 or DRIP205 and stimulated with 1,25D3 (10^{-8} M), butyrate (2 mM), or the combination. Again, silencing of SRC3 blocks induction of cathelicidin and CD14, whereas silencing of DRIP205 has no effect. HDAC inhibition by butyrate is not sufficient to reverse this effect. Data shown are means (±SD) of results from a single representative experiment performed in triplicates. These experiments were repeated using at least two different batches of primary keratinocytes to confirm reproducibility.

roles in disease pathogenesis, are currently unknown. Similarly, the therapeutic advantages of influencing this system in skin diseases including cutaneous bacterial infection are unexplored. In particular, targeting this system could be beneficial in situations where antimicrobial peptide expression is inadequate or where protective expression could be therapeutically induced such as in wounds (Ong *et al.*, 2002; Schauber *et al.*, 2007). The goal of the present study was to further define the essential elements of vitamin D_3 regulation of keratinocyte antimicrobial defense.

We found that increased histone acetylation enhanced 1,25D3-induced innate defense genes and increased antimicrobial activity in keratinocytes. This effect was mediated through the VDR and dependent on SRC3. Blocking or silencing of the VDR or SRC3 inhibited 1,25D3-induced cathelicidin and CD14 gene expression. HDAC inhibition by butyrate could not reverse this effect. This suggests that chromatin opening through HAT activity, mediated by the SRC3 complex, is necessary before the effects of VDR-mediated transcription can be enhanced by HDAC inhibition. Surprisingly, deletion of DRIP205 mediator did not prevent 1,25D3 induction of cathelicidin and CD14. To our knowledge this is the only example of gene induction by 1,25D3 independent of DRIP205, suggesting that unraveling of the chromatin via histone acetylation plays a much more important role than activation of the RNA polymerase via this mediator.

TLR2 ligands such as Malp-2 or PAM3CSK were shown here to greatly increase the acetylation of histone 4 in keratinocytes. When cells were stimulated with both TLR2/6 ligands and 1,25D3, they show the greatest increase in cathelicidin (Schauber *et al.*, 2007). This response is similar to the increased cathelicidin seen after HDAC and 1,25D3 treatment. Combined with the observations of the inhibition of expression by suppression of SRC3, these data strongly suggest histone acetylation is a modifier of 1,25D3-dependent effects. However, increased histone acetylation alone did not greatly induce cathelicidin or influence expression of other immune response genes such as IL-8. Since TLR2 activation by Malp-2 or PAM3CSK can induce IL-8, this

suggests histone acetylation cannot be the sole mechanism for the induction of either antimicrobial peptide expression or IL-8 production. These findings do, however, suggest that histone acetylation in keratinocytes can explain the amplification of an antimicrobial response to TLR2 ligands in the presence of 1,25D3 (Schauber *et al.*, 2007). Other cell types have distinct responses. TLR-mediated gene expression in macrophages and dendritic cells can be modified by HDAC inhibition (Brogdon *et al.*, 2007), and colonic epithelial cells can be induced to increase cathelicidin by HDAC alone (Schauber *et al.*, 2006). These observations show that the nature of a response to histone acetylation will be cell-type and gene-specific.

The observations presented in this study provide early evidence for a new mechanism in the regulation of innate immune function in skin and might lead to new treatments. Various HDACi have already been evaluated preclinically as therapeutic agents with potential roles in cancer treatment or as anti-inflammatory drugs (Konstantinopoulos *et al.*, 2007). Also, Raqib *et al.* (2006) were able to demonstrate that the HDAC inhibitor butyrate was beneficial in the treatment of experimental *Shigellosis*. *Shigella* bacteria are able to down-regulate cathelicidin expression in colonic epithelial cells and invade the mucosa (Islam *et al.*, 2001). These bacteria have evolved mechanisms to exploit epigenetic signals to repress innate immunity. *Shigella* bacteria inject effector proteins into host cells, thereby, inhibiting histone modification that leads to the suppression of defense gene induction (Arbibe *et al.*, 2007). Loss of cathelicidin expression can be reversed by oral administration of the HDAC inhibitor butyrate and contributes to rapid clearing of *Shigella* leading to clinical improvement (Raqib *et al.*, 2006). Yet, the potential therapeutic role for HDACi in strengthening and fine-tuning innate immune function in skin has not been addressed. HDACi could possibly be used in combination with 1,25D3 or its analogs to prevent or treat cutaneous infections. Alternatively, HDACi treatment could be combined with widely used UVB irradiation, which stimulates endogenous production of 1,25D3 to increase the innate immune defense barrier (Bar *et al.*, 2007). Recently, the 1,25D3 analog Maxacalcitol (1,25OH_2-22-oxacalcitriol) has shown promise in the treatment of viral infections of the skin (Imagawa and Suzuki, 2007). However, it remains unclear if treatment with 1,25D3 alone, or in combination with HDACi, is beneficial in the treatment of bacterial skin infections. Currently, vitamin D_3 analogs are also successfully used in the treatment of non-infectious inflammatory skin diseases such as psoriasis. Our results suggest that in combination therapy with HDACi, 1,25D3 dosages could possibly be lowered and the risk of 1,25D3-mediated side effects be minimized, or the beneficial activity of existing topical 1,25D3 treatments might be enhanced. Further clinical studies are required to investigate if disorders treated with 1,25D3 analogs may benefit from additional HDACi treatment alone or in combination with 1,25D3.

Before HDACi can be used for influencing innate immune function, their molecular effects and also risks have to be characterized very carefully. Histone modifications might, for example, not be beneficial in clinical situations such as human papilloma virus infection. In particular, HPV16 E7 has evolved a mechanism to increase histone acetylation and create a transcriptionally active chromatin structure to facilitate viral replication in cutaneous host cells (Longworth and Laimins, 2004; Longworth *et al.*, 2005). In this situation, additional pharmaceutical HDAC inhibition might benefit the pathogen rather than the host. Also, inhibition of histone deacetylation by TSA promotes abnormal epidermal differentiation and disturbed epidermal homeostasis in organ cultures of human foreskin (Markova *et al.*, 2007). Furthermore, pharmacological studies on the ability of different HDACi to penetrate the skin have not been reported. Finally, HDACi increase VDR-mediated transcriptional activity by aiding the function of SRC3/p300 acetyltransferase. SRC family members are also involved in the activity of other nuclear receptors such as RXR, LXR, and PPAR, which play vital roles in epidermal differentiation and cutaneous physiology. The effects of HDAC inhibition on the function of those nuclear receptors are to date not known.

Taken together, this study defines previously unknown elements that regulate innate immune function of keratinocytes and demonstrates the potential for a novel approach to immune therapy through epigenetic modification of histone acetylation. On the basis of these observations, it will be important to investigate if disturbed antimicrobial defense in the skin or other disorders characterized by altered innate immune responses are influenced by histone acetylation and may benefit by targeted therapy to this pathway.

MATERIALS AND METHODS

Cell culture and stimuli

NHEKs were grown in serum-free EpiLife cell culture media (Cascade Biologics, Portland, OR) containing 0.06 mM Ca^{2+} and 1 × EpiLife defined growth supplement at 37°C under standard tissue culture conditions. Stock cultures were maintained for up to four passages in this media with the addition of 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. HaCaT keratinocytes were cultured in DMEM (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin. Cells at 50–70% confluence were stimulated with 1,25D3 (10^{-9}–10^{-7} M; Sigma, St Louis, MO), butyrate (2 mM; Sigma), or their combination for 24 hours. In addition, NHEKs were stimulated with the HDACi TSA (200 ng ml^{-1}; Sigma) or TLR ligands PAM3CSK (1 μg ml^{-1}; Invivogen; San Diego, CA), Malp-2 (0.1 μg ml^{-1}; Alexis, Carlsbad CA), poly(I:C) (25 μg ml^{-1}; Amersham, Piscataway, NJ), lipopolysaccharide (1 μg ml^{-1}; Sigma), Flagellin (50 ng ml^{-1}; Alexis), Imiquimod (10 μg ml^{-1}; Invivogen), or CL075 (5 μg ml^{-1}; Invivogen). Furthermore, NHEKs were pretreated with a vitamin D receptor antagonist ZK159222 (provided by Dr Zügel from Schering AG; 10^{-7} M) before stimulation with 1,25D3 (10^{-8} M) in the presence of butyrate (2 mM).

Real-time RT-PCR

After cell stimulation, total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and 1 μg RNA was reverse transcribed using iScript (BioRad, Hercules, CA). The expression of cathelicidin was evaluated using an FAM-CAGAGGATTGTGACTTCA-MGB

probe with primers 5′-CTTCACCAGCCCGTCCTTC-3′ and 5′-CCAG GACGACACAGCAGTCA-3′. For glyceraldehyde-3-phosphate dehydrogenase expression, a VIC-CATCCATGACAACTTTGGTA-MGB probe with primers 5′-CTTAGCACCCCTGGCCAAG-3′and 5′-TGG TCATGAGTCCTTCCACG-3′ was used as described (Schauber *et al.*, 2006). Predeveloped Taqman assay probes (Applied Biosystems ABI, Foster City, CA) were used for the analyses of the expression of CD14, CYP24A1, and IL-8. All analyses were performed in triplicate from 2 to 5 independent cell stimulation experiments in an ABI Prism 7000 Sequence detection system. Fold induction relative to the vehicle treated control was calculated using the equation $2^{(-\Delta\Delta C_t)}$ where ΔC_t is $\Delta C_{t(\text{stimulant})} - \Delta C_{t(\text{vehicle})}$, ΔC_t is $C_{t(\text{cathelicidin})} - C_{t(\text{GAPDH})}$, and C_t is the cycle at which the detection threshold is crossed. Results were considered significant when at least a three-fold difference in expression levels was detected and statistical analysis revealed *P*-values <0.05.

Fluorescence immunohistochemistry

NHEKs were grown on chamber slides and stimulated with 1,25D3, butyrate, or various TLR ligands for 24 hours. After acetone fixation and subsequent washings in phosphate-buffered saline, slides were blocked in 3% BSA in phosphate-buffered saline for 30 minutes at RT and stained with a polyclonal chicken anti-hCAP-18/LL-37 (Schauber *et al.*, 2006) or anti-acetylated histone H4 primary antibody (Cell Signaling, Danvers, MA) or preimmune serum. After washings in phosphate-buffered saline, slides were reprobed with an FITC-labeled goat anti-chicken antibody. After subsequent washings with phosphate-buffered saline, slides were mounted in ProLong Anti-Fade reagent containing 4,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR) and evaluated with an Olympus BX41 microscope (Olympus, Melville, NY) at $\times 400$ or $\times 600$ original magnification.

RNAi and transfection

Second passage keratinocytes derived from neonatal human foreskin were maintained in serum-free keratinocyte growth medium creatine kinase (Cascade Biologics) containing 10 ng ml^{-1} hrEGF (half the dose compared to Cascade's standard composition). Keratinocytes were transfected with 20 nM siRNA oligonucleotides using siLentFect (BioRad) with creatine kinase media. A mixture of four siRNA oligonucleotides for each VDR (Dharmacon, Lafayette, CO; SMART Pool) and DRIP205 (Dharmacon; PPARBP SMART Pool), a single oligonucleotide for SRC3 (Qiagen, Valencia, CA; AIB1 pub 1024591), and non-targeted control siRNA (Dharmacon) were used. The cells were transfected twice (24 hours a part) to maximize the silencing effect. The efficiency of blockage was evaluated by real-time RT-PCR (qPCR) and/or western analysis as described (Oda *et al.*, 2003). Blockage efficiency in these experiments was 66% for siVDR, 82% for siDRIP205, and 87% for siSRC3 compared to baseline. Expression of cathelicidin and CD14, and the housekeeping gene L19 transcript abundance was analyzed in siRNA-transfected cells by real-time qPCR using SYBR green as described (Oda *et al.*, 2007). Primer sets for cathelicidin (CAMP) (CAMP417F, 5′-TCACCAGAGGATTGTGACTTCAA-3′; CAMP546R, 5′-CCAGC AGGGCAAATCTCTTG-3′), VDR (hVDR1098F, 5′-GAGGAGCAT GTCCTGCTCATG-3′; hVDR1157R, 5′-CTGCACCCCAGGACGATCT-3′), DRIP205 (DRIP205F, 5′-TCCCCTAGCATTAAAGCCAAAGTG-3′; DRIP205R, 5′-CTTCCTCCCCAATCATAAAGTCTGG-3′), SRC-3 (hRAC3F, 5′-CAGCCCACCTCCTAATGTGACTG-3′; hRAC3R, 5′-TTTCTGATCAGGACCCATAGGCA-3′, and CD14 (CD14 314F, 5′-GAGCCTGTCCGGAGCTCA-3′; CD14 443R, 5′-TCATCGTCCA GCTCACAAGG-3′) were designed using Primer Express (ABI) to span exon–intron boundaries. These experiments were repeated using at least two different batches of primary keratinocytes to confirm reproducibility.

Western blot

Keratinocytes were stimulated with butyrate (2 mM), various TLR ligands, or the vehicle for 24 hours and subsequently lysed in ice-cold RIPA buffer containing proteinase inhibitors (Roche, Indianapolis, IN). After centrifugation, equal amounts of protein were mixed with loading buffer (0.25 M Tris-HCl, 10% SDS, 10% glycerol, 5% β-mercaptoethanol) and loaded onto a 16% Tris-Tricine gel (GeneMate, Kaysville, UT). After separation, proteins were blotted onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA) and blocked in 5% milk (BioRad) in Tris-buffered saline 0.1% Tween-20 for 1 hour at RT. After washing in Tris-buffered saline 0.1% Tween-20, membranes were stained with a rabbit polyclonal anti-acetylated histone H4 antibody (Cell Signaling) or a mouse monoclonal anti-CD14 antibody (R&D Systems, Minneapolis, MN), washed again in Tris-buffered saline 0.1% Tween-20 and reprobed with a HRP-coupled goat anti-rabbit antibody (DakoCytomation, Glostrup, Denmark). Stained protein was visualized using the Western Lightning system (Perkin Elmer, Boston, MA). Densitometric analyses were performed using ImageJ version 1.37r. The blockage of VDR and coactivator protein expressions was also confirmed by western blot analysis. Nuclear extracts were prepared from siRNA-transfected cells (six-well plate) using NE-PCR nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) according to the manufacturer's instructions. Equal amount of proteins (20 μg nuclear extracts) were separated by SDS–PAGE and electroblotted onto a polyvinylidene difluoride membrane. After blocking, the blot was incubated with mouse monoclonal antibody against VDR (Santa Cruz D-6, Santa Cruz, CA), goat polyclonal antibody against DRIP 205 (Santa Cruz TRAP220 C-19), or rabbit polyclonal antibody against SRC3 (Affinity Bioreagents, Golden, CO; PA1-845). Subsequently, the blot was incubated with appropriate secondary antibody conjugated with HRP (Amersham), and bound antibody was visualized using a chemiluminescence system (SuperSignal ULTRA; Pierce).

Immunohistochemistry

Immunohistochemistry was performed to detect localization of cathelicidin, DRIP205, and SRC3 in the epidermis. Paraffin sections of human adult skin were incubated with antibodies against DRIP205 (Santa Cruz) and SRC3 (Affinity Bioreagents PA1-845) (Oda *et al.*, 2003). The sections were then incubated with biotinylated secondary antibody, followed by alkaline phosphatase-conjugated ABC complex (Vector, Burlingame, CA). The signals were visualized with NBT/BCIP substrate (Roche). All sample acquisitions were approved by the committees on investigations involving human subjects at the Veterans Affairs Medical Center, San Francisco.

Antimicrobial assay

Antimicrobial activity of stimulated and unstimulated epithelial cells was determined using a modified protocol described in Schauber

et al. (2006). Cells were grown and stimulated without antibiotics, harvested in 100 µl sterile water and sonicated on ice for 20 minutes. For solution killing assays, *S.* ΔmprF (Peschel *et al.*, 2001) was grown in tryptic soy broth (Sigma) overnight and then subcultured in 20% tryptic soy broth, 25 mM $NaHCO_3$, and 1 mM $NaHPO_4$ until log phase. Twenty thousand bacteria ($OD_{600} = 1.0$ corresponds to 3.75×10^9 colony-forming units per ml) were incubated with various cell lysate concentrations at 37°C in 20% tryptic soy broth, 25 mM $NaHCO_3$, and 1 mM $NaHPO_4$. Bacterial growth over time was determined by measuring OD at 600 nm.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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